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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Brian Seed et al.	Art Unit:	1644
Serial No.:	08/756,018	Examiner:	G. Ewoldt
Filed:	November 25, 1996	Customer No.:	21559
Title:	P-SELECTIN LIGANDS AND RELATED MOLECULES AND METHODS		

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APPELLANTS' BRIEF ON APPEAL
SUBMITTED PURSUANT TO 37 C.F.R. § 41.37

In support of Appellants' Notice of Appeal that was filed in the above-captioned case on May 31, 2002, of the Examiner's final rejection mailed on December 7, 2001, submitted herewith is Appellants' Brief on Appeal.

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Regents of the University of California v. Eli Lilly & Co., 119 F.3d 1559, 1569 (Fed. Cir. 1997).

Real Party in Interest

The real party in interest is The General Hospital Corporation, to whom all interest in the present application has been assigned. (Reel 8437, Frame 0899).

Related Appeals and Interferences

There are no currently pending appeals or interferences related to this case.

Status of Claims

Claims 1-9, 11, and 15-23 are canceled.

Claims 10, 12-14, and 24-25 are currently pending.

Claims 10, 12-14, and 24-25 were rejected in a Final Office Action mailed on December 7, 2001, and are appealed.

Status of Amendments

All amendments have been entered.

Summary of Claimed Subject Matter

Applicants have discovered that it is possible to create artificial P-selectin ligands by combining amino acid sequences containing tyrosine sulfation sites with sialyl Le^x addition sites where at least one of the sites is positioned at a non-naturally occurring

site on the protein (page 2, lines 19-21, and Figure 14). These sites may originate in different polypeptides and be inserted into a third, carrier polypeptide, or the sites may originate from the same polypeptide and be repositioned relative to one another (page 3, lines 7-9). The P-selectin ligands may further include, or be fusion proteins with, an antibody (page 3, line 1-3, and page 27, line 13 through page 28, line 4).

As presently claimed, the invention features purified nucleic acids that encode an artificial P-selectin ligand polypeptide that contains a tyrosine sulfation site and a sialyl Le^x addition site, wherein at least one of the sites is located at an amino acid position where it does not naturally occur (page 3, lines 4-9 and, for example, page 16, lines 5-21 and Figure 14). The invention also features vectors and cells containing the claimed nucleic acids (page 3, line 9).

Grounds of Rejection to Be Reviewed on Appeal

This appeal presents two issues:

1. Whether the Examiner erred in rejecting claims 10, 12-14, and 24-25 under 35 U.S.C. § 112, first paragraph, for lack of enablement.

2. Whether the Examiner erred in rejecting claims 10, 12-14, and 24-25 under 35 U.S.C. § 112, first paragraph, for inadequate written description.

ARGUMENT

Issue 1: Rejection of Claims 10, 12-14, and 24-25 for Lack of Enablement

The Patent Office has the initial burden to establish a reasonable basis to question enablement. In *In re Marzocchi*, 439 F.2d 220, 169 U.S.P.Q. 367, 369 (C.C.P.A. 1971), the court stated:

> a specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as in compliance with the enabling requirement of the first paragraph of § 112 unless there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support.

The M.P.E.P. (§ 2164.04) echoes the holding of *Marzocchi*:

> (I)t is incumbent upon the Patent Office, whenever a rejection on this basis is made, to explain why it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement. Otherwise there would be no need for the Appellant to go to the trouble and expense of supporting his presumptively accurate disclosure.

Claims 10, 12-14, and 24-25 were finally rejected under 35 U.S.C. § 112, first paragraph, for lack of enablement on two grounds. First, the Examiner asserts that (Paper 34, Final Office Action mailed December 7, 2001, page 3):

> Applicants' disclosure of a single polypeptide in support of the broad genus encompassed by Claim 10 is insufficient support that additional polypeptides could be made without undue experimentation

because

> the disclosure is insufficient to support the instant claims to an invention that would encompass an essentially unlimited number of polypeptides.

Second, the Examiner, after pointing out that the specification discloses experimental results from *in vitro* assays while the intended uses are *in vivo* and, recognizing that working examples are not a criterion for patentability, asserts that "some sort of *in vivo* enablement is required." The Examiner argues that:

> the specification fails to establish sufficient correlation between said assay and any *in vivo* process, thus, said assay can not be considered a relevant *in vitro* model for any *in vivo* process.

Applicants respectfully disagree with these grounds of rejection and submit that the Examiner has failed to rebut the presumption that Applicants' disclosure is accurate and enabling.

A Large Genus Does Not Prima Facie Lack Enablement

The Examiner asserts that undue experimentation is required to practice the claimed invention because there are "an essentially unlimited number of tyrosine sulfation sites" resulting in the claims encompassing an "essentially unlimited number of polypeptides." The Examiner also asserts that Applicants have not addressed the problem

of the positioning of the sialyl Le^x addition sites and tyrosine sulfation sites in relation to one another. These assertions are in error.

Tyrosine Sulfation Sites

The standard for enablement is articulated in *In re Wands* 858 F.2d 731, 8 U.S.P.Q.2d 1400 (Fed. Cir. 1988). In defining the boundaries of undue experimentation, the *Wands* court stated that "the key word is 'undue' not 'experimentation'" and that "the test is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine." *Id.* at 737.

In asserting this argument, the Examiner has improperly concluded that the disclosure must not be enabling because the size of the claimed genus is large. Specifically, the Examiner asserts that the number of possible tyrosine sulfation sites is "essentially unlimited." Applicants respectfully disagree and first point out that, regardless of the size of a claimed genus, a disclosure is enabling if an artisan is able to practice the full scope of that genus.

As discussed in Applicants' Reply of September 20, 2001, and reasserted here, the characteristics of tyrosine sulfation sites were well recognized in the art at the time of application filing. A skilled artisan would have no trouble in making and using such sites in a synthetic P-selectin ligand. For example, Hortin *et al.* (*Biochem. Biophys. Res. Comm.*,

141:326-333, 1986) and Huttner (*Annu. Rev. Physiol.*, 50:363-376, 1988) had extensively characterized the requirements for tyrosine sulfation. Hortin *et al.* state (page 331):

> Based on the foregoing analysis of amino acid sequences surrounding sulfation sites, **five simple rules** were empirically derived to aid in predicting the location of sites of sulfation. Tyrosine residues that are likely sites of sulfation are identified by the following criteria:
> 1) There is an acidic residue at position -1 or -2,
> 2) There are at least 3 acidic amino acid residues within 5 residues (positions -5 to +5) of the tyrosine residue,
> 3) No more than 1 basic amino acid residue are within 5 residues of the tyrosine,
> 4) No more than 3 hydrophobic residues (Ile, Leu, Phe, and Val) are within 5 residues of the tyrosine,
> 5) No cysteine residues are within 15 residues of the tyrosine.

Hortin makes it clear that there are ten critical amino acids, other than the tyrosine, which comprise a sulfation site (criteria 1-4). Although one can "imagine" a large number of ten amino acid combinations, a formulaic application of the "five simple rules" will immediately provide the artisan with an expectation of success or failure. The "trial-and-error" in tyrosine sulfation site design is carried out largely by testing a proposed amino acid sequence against the "five simple rules" and eliminating those which do not conform.

Further, Applicants provide the artisan with several tyrosine sulfation sites to use intact, or as a starting point for further modification. Specifically, Applicants provide extensive characterization of the tyrosine sulfation site of PSGL-1 (see, Specification page 21, line 11 through page 23, line 14). Applicants also identify the tyrosine sulfation site of

coagulation Factor VIII (SEQ ID NO: 15). Thus, using Applicants' disclosure and information known in the art at the time of filing, synthetic tyrosine sulfation sites and P-selectin ligands could be designed with a high expectation of success before ever entering the laboratory. The only experimentation remaining is routine screening to determine the effectiveness of the synthetic P-selectin ligand, assays for which are provided in Applicants' specification at page 13, line 11 through page 16, line 4. Applicants respectfully submit that, regardless of the number of possibilities, the design of tyrosine sulfation sites does not require undue experimentation.

Of course, this ground of rejection is moot insofar as applies to claim 24 (Group 2) because the claim specifically recites the tyrosine sulfation site of Factor VIII provided in SEQ ID NO: 15. Thus, claim 24 does not encompass a "genus" of tyrosine sulfation sites.

Positioning of the Sialyl Le^x Addition Site and Tyrosine Sulfation Site

The Examiner further asserts that "Applicant has not addressed the problem of positioning said sialyl Le^x addition sites and tyrosine sulfation sites in relation to one another." This assertion is clearly incorrect and does not support the enablement rejection.

As noted previously (Reply of September 20, 2001), part of Applicants' inventive contribution is the recognition that, for a synthetic P-selectin ligand, the relationship (conformation and distance) between the tyrosine sulfation site and the sialyl

Le^x site is more flexible than previously thought. Applicants' disclosure demonstrates that non-naturally occurring P-selectin binding molecules can be made without strictly inserting a naturally occurring P-selectin recognition site. Thus, Applicants have demonstrated that greater flexibility for designing artificial proteins exists.

Further, contrary to the Examiner's assertion, Applicants provide guidance on the relative positioning and intervening sequence requirements between the tyrosine sulfation and sialyl Le^x sites. For example, in Figure 3, Applicants demonstrate that fusion of the N-terminal domain of PSGL-1, containing the tyrosine sulfation site, facilitates P-selectin binding activity in chimeric molecules having a sialyl Le^x site derived from any of CD43, CD34, or GlyCAM-1. These experiments show that the P-selectin binding is tolerant of considerable sequence variability in the region linking the tyrosine sulfation and the sialyl Le^x sites. Applicants also constructed mutant PSGL molecules by varying the number of repeated elements. This has the effect of altering the spacing between the tyrosine sulfation and the sialyl Le^x sites. Figure 4 of Applicants' specification demonstrates that P-selectin binding function is maintained despite considerable deletion of the repeated elements in PSGL. As shown in Figure 4B, deletion of 1-3 repeated elements did not appreciable affect P-selectin binding, and mutants having a deletion of 4-8 elements retained approximately 75% of the native binding activity. Thus, contrary to the Examiner's assertion, Applicants have addressed the problem of positioning of the sialyl

Le^x and the tyrosine sulfation sites relative to one another and have presented this information in their specification.

Working Examples

Finally, on this issue, Applicants wish to address the Examiner's incorrect assertion that Applicants provide only a single working example (Figure 13) in the specification. To the contrary, in addition to the HL-60 cell rolling assay (described in more detail below), Applicants demonstrate the P-selectin binding activity of synthetic P-selectin ligands. For example, Figure 3B shows that COS cells expressing a synthetic P-selectin ligand that is a chimera of PSGL-1 and either CD43 or CD34 possess P-selectin binding activity equal to COS cells expressing native PSGL-1. And a chimera of PSGL-1 and GlyCAM-1 is shown to impart a binding activity of approximately 50% compared to native PSGL-1.

The results presented in Figure 4 also constitute a working example of the claimed nucleic acids. Specifically, the nucleic acids encoding the PSGL-1 deletion mutants of Figure 4 fall within the scope of claim 10 because the encoded polypeptides contain both an N-linked sialyl Le^x addition site and a tyrosine sulfation site wherein at least one of the sites is located at a non-naturally occurring position. As described above, several of these deletion mutants demonstrate P-selectin binding activities that are equivalent to native PSGL-1.

Finally, in Figure 15 of the specification, Applicants show that COS cells expressing a chimera of CD43 and Factor VIII also have a high level of P-selectin binding activity. Thus, contrary to the Examiner's assertion, Applicants have provided more than a single working example. Applicants have, in fact, made a significant showing of data in the form of working examples which enable the artisan to fully practice the claimed invention.

The Specification is Enabling for In Vivo Uses

As a second basis for the enablement rejection, the Examiner asserts that the specification discloses *in vitro* assay results but "the only specific disclosed intended uses are *in vivo*." The Examiner further states that (Paper 34: Final Office Action of December 7, 2001; page 3, last sentence):

> the specification fails to establish sufficient correlation between said assay and any *in vivo* process, thus, said assay can not be considered a relevant *in vitro* model for any *in vivo* process.

Applicants respectfully disagree as this conclusion is in error.

As noted previously, the Federal Circuit and the Patent Office provide Applicants with the presumption that a disclosure is enabling. *In re Marzocchi*, 439 F.2d 220, 169 U.S.P.Q. 367, 369 (C.C.P.A. 1971); M.P.E.P. § 2164.04. The Examiner is required to explain why he doubts the truth or accuracy of any statement in a supporting disclosure and to back up those assertions with acceptable evidence or reasoning which is

inconsistent with the contested statement. In the present case, the Examiner has done nothing other than provide an unsubstantiated conclusory statement of doubt. Nowhere does the Examiner provide evidence or reasoning by which he arrives at this conclusion.

The M.P.E.P. § 2164.02 sets forth the requirements for enablement with regard to working examples. In relevant part, § 2164.02 states:

> An *in vitro* or *in vivo* animal model example in the specification, in effect, constitutes a "working example" if that example "correlates" with a disclosed or claimed method invention.
>
> *****
>
> In this regard, the issue of "correlation" is also dependent on the state of the prior art. In other words, if the art is such that a particular model is recognized as correlating to a specific condition, then it should be accepted as correlating unless the examiner has evidence that the model does not correlate.
>
> *****
>
> A rigorous or an invariable exact correlation is not required, as stated in *Cross v. Iizuka*, 753 F.2d 1040, 1050, 224 U.S.P.Q. 739, 747 (Fed. Cir. 1985).

Thus, the M.P.E.P. recognizes that *in vitro* assays can support claims to *in vivo* utilities if there is a correlation between the assay and the *in vivo* condition. The M.P.E.P. also notes that the specification alone need not establish the correlation provided that a correlation was known at the time of application filing.

The Federal Circuit recognizes the position articulated in M.P.E.P. § 2164.02, that claims to *in vivo* applications may be supported by experimental results from *in vitro* assay systems. The court in *In re Brana,* 51 F.3d 1560. 34 U.S.P.Q.2d 1436 (Fed. Cir. 1995) reversed a U.S.P.T.O. decision that *in vitro* activity did not support therapeutic

applications *in vivo*. The appellants in *In re Brana* used *in vitro* data from two lymphocytic leukemia cell lines to support claims to *in vivo* uses of chemotherapeutic agents. Similarly, Applicants demonstrate *in vitro* efficacy of synthetic P-selectin ligands using an HL-60 (human leukemia cell line) cell rolling assay (Figure 13 and page 15) to support *in vitro* diagnostic and *in vivo* therapeutic uses. Thus, Applicants' claims do not lack enablement, *per se*, because no *in vivo* animal or human testing data is disclosed.

The HL-60 cell rolling assay correlates with *in vivo* inflammatory processes and is sufficient to support *in vivo* utilities for the synthetic P-selectin ligands of the invention. The correlation between leukocyte rolling and the inflammatory process *in vivo* were well documented in the art at the time of filing. For example, Kubes *et al.* state (*J. Immunol.*, 152:3570-3577, 1994, first paragraph, first sentence; emphasis added; art of record):

> The movement of leukocytes from the main stream of blood to afflicted tissue is a key feature of inflammation. This process consists of at least two distinct events: 1) the initial contact between the leukocyte and endothelium described as leukocyte rolling and 2) firm or stationary adhesion.

The art also identifies both *in vitro* and *in vivo* rolling assays using various leukocyte cell types, including the HL-60 promyelocytes used by Applicants. These rolling assays were recognized and used as a reliable indicator and investigational tool for the early stages of the inflammatory process.

The law is well settled that *in vitro* assay results can support claims to *in vivo* utilities provided that there is a reasonable correlation between the two. Both the specification and the prior art understood that the process of leukocyte rolling is an integral part of the inflammatory process *in vivo*. *In vitro* cell rolling assays generally, and the HL-60 cell rolling assay used by Applicants, were recognized as useful surrogates for investigating *in vivo* inflammatory processes. As the Examiner has provided no evidence to the contrary, Applicants respectfully submit that there is a strong correlation between the *in vitro* HL-60 rolling assay and the *in vivo* inflammatory process. Accordingly, the results provided in the specification constitute working examples and provide the artisan with a reasonable expectation of success in using the synthetic P-selectin ligands of this invention.

Summary

In view of the above, Applicants submit that the present specification enables the skilled artisan to make and use the synthetic P-selectin ligands encoded by the nucleic acids of the present invention. In view of Applicants' disclosure, the artisan is able to recognize, create, and modify both the tyrosine sulfation sites and sialyl Le^x addition sites, as well as alter the spacing between the two, with a reasonable expectation of retaining P-selectin binding. Further, Applicants' demonstration that synthetic P-selectin ligands block HL-60 rolling provides an expectation that these ligands will be successful as

diagnostics and therapeutics for *in vitro* or *in vivo* use. Accordingly, the rejection of claims 10, 12-14, and 24-25 for lack of enablement should be reversed.

Issue 2: Rejection of Claims 10, 12-14, and 24-25 for Inadequate Written Description

In asserting this rejection, the Examiner states that

> [t]he specification discloses no limitation on the sites which might comprise either a "sialyl Le^x addition site" or a "tyrosine sulfation site". Absent any disclosed limitations, a "sialyl Le^x addition site" must be considered to be any amino acid capable of accepting either an O-linked or N-linked carbohydrate addition. Similarly, a "tyrosine sulfation site" must be considered to be any tyrosine. Paper 32: Office Action of March 21, 2001, page 4.

Applicants respectfully disagree and point out that, for the reasons discussed previously, a tyrosine sulfation site is not any tyrosine, nor is a sialyl Le^x addition site any amino acid capable of accepting either an O-linked or N-linked carbohydrate addition. Applicants also note that, contrary to the Examiner's assertion, more than a single functional example of each of these sites is disclosed.

The Federal Circuit, in *Regents of the University of California v. Eli Lilly & Co.*, 119 F.3d 1559, 1569 (Fed. Cir. 1997), provide for fulfillment of the written description requirement either through the enumeration of a plurality of species or by "other appropriate language." As an alternative to reciting a large number of species falling within a claimed genus, the *Lilly* court specifically provides for "a recitation of structural features

common to the members of the genus, which features constitute a substantial portion of the genus." *Id.* at 1569.

Applicants respectfully submit that ample guidance and description is provided by the specification or was known in the art at the time of filing to provide an adequate written description of the claimed invention.

Sialyl Le^x Addition Sites

Contrary to the Examiner's assertion, a sialyl Le^x addition site cannot be "any amino acid capable of accepting either an O-linked or N-linked carbohydrate addition." Applicants point out that claim 10 is limited, in relevant part, to nucleic acids encoding polypeptides containing N-linked sialyl Le^x addition sites. The specification describes N-linked glycan addition as occurring at the consensus site of N X S/T, wherein N is asparagine, S is serine, T is threonine, and X is any amino acid except proline. Specification at page 25, lines 20-22. Applicants also provide other guidance for creating and identifying sialyl Le^x addition sites. For example, Figure 10 identifies at least five sites for N-linked glycan addition in IgG1. Further guidance is provided in the specification at page 25, line 22 through page 26, line 17. Preferable sites are present or created in the CH2 region of an immunoglobulin molecule, are located on the outside of the immunoglobulin molecule, or are located in a region which is minimally disruptive to the primary and

secondary structure of the protein. Thus, an artisan is clearly apprised of the structural features of a sialyl Le^x addition site and where such sites should be created.

Moreover, this ground of rejection is moot insofar as it applies to claim 25 (Group 3) because the claim specifically recites the nucleic acids encoding a polypeptide containing Ile135 through Ser225 of the CD43 precursor sequence (SEQ ID NO: 17). The sialyl Le^x addition site recited in claim 25 is therefore explicitly described.

Tyrosine Sulfation Sites

The Examiner is also incorrect in asserting that "a tyrosine sulfation site must be considered to be any tyrosine." As discussed previously, sulfation occurs at tyrosines that are contained within sequences of a very specific character and for which tests are available in the art. Further, Applicants provide several examples of tyrosine sulfation sites useful in creating the synthetic P-selectin ligands of this invention. For example, Figure 14 describes the tyrosine sulfation site of coagulation Factor VIII and the fourth component of human complement. Additionally, Applicants demonstrate the tolerance of the PSGL-1 sulfation site to modification. In Figure 9 and the accompanying text at page 22, line 7 through page 23, line 4, Applicants provide experimental data on the biological effects of altering the tyrosine sulfation site. Specifically, Applicants demonstrate that conversion of the tyrosines to phenylalanines results in the complete loss of P-selectin binding, whereas

replacement of the threonine residues with alanine reduced but did not abolish binding activity. Thus, the specification and the prior art clearly describe tyrosine sulfation sites.

In addition, this ground of rejection is moot insofar as it applies to claim 24 (Group 2) because the claim specifies nucleic acids encoding a polypeptide that contains the tyrosine sulfation site of Factor VIII as provided in SEQ ID NO: 15. The tyrosine sulfation site recited in claim 24 is, therefore, explicitly described.

Positioning of the Sialyl Le^x Addition Site and Tyrosine Sulfation Site

For the reasons discussed previously, Applicants submit that the relative positioning of the sialyl Le^x addition site and tyrosine sulfation site is adequately described in the specification. Specifically, in Figure 4, Applicants describe the production and biological activity of PSGL mutants with altered spacing between these two sites. Site spacing was altered by deleting a varying number of the repeated elements of PSGL. Additionally, Applicants describe artificial P-selectin ligands that are combinations of sialyl Le^x addition sites and tyrosine sulfation sites derived from different molecules (see, for example, Figure 3). These synthetic ligands also have varied spacing between the two sites. Applicants submit that, on the issue of the spacing between the sialyl Le^x addition site and tyrosine sulfation site, the genus of synthetic P-selectin ligands encoded by the claimed nucleic acids is adequately described in the specification.

Summary

Applicants respectfully submit that the specification clearly describes sialyl Le^x addition sites, tyrosine sulfation sites, and their spacing in artificial P-selectin ligands. Contrary to the Examiner's assertion, neither of these sites encompasses an unlimited number of possibilities. Accordingly, the rejection of claims 10, 12-14, and 24-25 for inadequate written description should be reversed.

CONCLUSION

Appellants respectfully request that the rejections of claims 10, 12-14, and 24-25 be reversed. No fee is believed due at this time.

If there are any additional charges or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

Date: 22 June 2006

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Appendix of Claims on Appeal

10. A purified nucleic acid encoding a polypeptide that is a synthetic P-selectin ligand, wherein said polypeptide contains an N-linked sialyl Le^x addition site and a tyrosine sulfation site, and wherein at least one of the sites is located at an amino acid position in said polypeptide which is different from its position in a naturally-occurring P-selectin ligand.

12. The purified nucleic acid of claim 10, wherein said nucleic acid further encodes an antibody or antibody fusion protein.

13. A vector comprising the nucleic acid of claim 10.

14. A cell comprising the nucleic acid of claim 10.

24. The purified nucleic acid of claim 10, wherein said tyrosine sulfation site consists of the Factor VIII tyrosine sulfation sequence set forth in SEQ ID NO: 15.

25. The purified nucleic acid of claim 10, wherein said polypeptide comprises Ile135 through Ser225 of the CD43 precursor sequence (SEQ ID NO: 17).

Appendix of Evidence

Exhibit	Evidence
I	Hortin et al., *Biochem. Biophys. Res. Comm.*, 141:326-333, 1986
II	Huttner (*Annu. Rev. Physiol.*, 50:363-376, 1998)

The references in Exhibits I and II were submitted to the Office in a Reply mailed June 1, 1999 (Paper No. 15). This Reply was acknowledged and entered by the Office in an action mailed August 31, 1999 (Paper No. 16).

EXHIBIT 1

Vol. 141, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 26, 1986 Pages 326-333

CHARACTERIZATION OF SITES OF TYROSINE SULFATION IN PROTEINS AND CRITERIA FOR PREDICTING THEIR OCCURRENCE

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SUMMARY: A wide variety of secretory proteins have recently been found to undergo post-translational sulfation of specific tyrosine residues. Here, amino acid sequences surrounding known sulfation sites in proteins are analyzed in order to identify factors which determine the specificity of sulfation. Several distinctive features of sulfation sites are identified, including: 1) abundance of acidic amino acid residues, 2) lack of basic residues, 3) low hydropathy, 4) absence of neighboring cysteine residues, 5) lack of extended secondary structure. Rules are proposed for predicting likely sites of sulfation based on the amino acid sequence of a protein.

Recent studies by Huttner and coworkers (1-3) indicate that sulfation of tyrosine residues is a common modification of secretory proteins. This post-translational modification occurs in a broad phylogenetic range of organisms, and a number of proteins in each tissue examined contain tyrosine sulfate (2, 3). Frequent occurrence of tyrosine sulfate was not recognized previously simply because this modified amino acid is acid-labile. Tyrosine sulfate is degraded by standard methods for compositional and sequence analysis of proteins (2, 4) so that its presence in a protein would not be detected, even if the protein has been sequenced in its entirety by standard techniques. Recognition of the widespread occurrence of the sulfation of proteins has stimulated efforts to identify proteins that contain this modification. Examples of human proteins recently found to contain tyrosine sulfate include: the fourth component of complement (4), fibronectin (5), α-fetoprotein (5), type III procollagen (6), α_2-antiplasmin (7, 8), and heparin cofactor II (9). The sulfation of tyrosine residues in proteins appears to be a highly selective process. Only specific tyrosine residues are modified in proteins that are substrates for sulfation. However, the structural determinants of the site specificity are not known. The present study analyzes amino acid sequences adjoining known sites of sulfation in order to clarify how specific tyrosine residues are recognized for sulfation.

0006-291X/86 $1.50

MATERIALS AND METHODS

Protein database: Amino acid sequences adjacent to 15 known sites of sulfation in 10 proteins were chosen as a database (Table I). The sources of amino acid sequences and identification of sites of sulfation are indicated. Sulfation sites have been determined in B-fibrinopeptides from a number of animal species (10), but, due to extensive homology of these sequences, only one was included in the database. Also, an assumption was made that small peptides known to contain tyrosine sulfate undergo sulfation while the peptides are still segments of larger precursor polypeptides. Validity of this assumption is supported by the observation that the single-chain precursor to C4 (4) and high-molecular weight forms of gastrin contain tyrosine sulfate (11).

Computer analysis: The computer program PARA-SITE[1] analyzes structural parameters as a function of distance from a specific index position (position 0). In this study, tyrosine sulfate residues are used as the index position. PARA-SITE calculates and plots the mean structural parameter value and the standard deviation for all amino acids at each distance from the index position. Secondary structure values used to describe α-helix, β-sheet, and β-turns were obtained from Chou and Fasman (12). Hydropathy values were from Kyte and Doolittle (13). PARA-SITE was written in the C programming language (14). All programs used in this report were run on a VAX 11/785 or MicroVAX II running VMS 4.4 (Digital Equipment Corporation).

RESULTS AND DISCUSSION

The amino acid sequences listed in Table I comprise the database used for analysis of sites of sulfation. No consensus sequence for sulfation sites is identified among these amino acid sequences. However, several distinctive features of these sequences surrounding sites of sulfation are evident. Most striking is the unusual distribution of several amino acids around sites of sulfation. The distribution of amino acids at different distances from tyrosine sulfate residues (designated as position 0) is tabulated in Table II. There is a very high concentration of acidic amino acid residues in the segment extending 5 residues either side of tyrosine sulfate residues. Within this segment, 46% of the residues are aspartic or glutamic acid, and each example in the database contains at least 3 acidic residues. The strongest preference for acidic residues is at the -2 and -1 positions, where 73% of all amino acids are aspartic or glutamic acid. Each example in the database contains at least one acidic residue at these two positions. In contrast, there are few basic residues in the -5 to +5 region. Only 3% of the total consists of basic residues, and no site of sulfation has more than one basic residue in this segment. Beyond the limits of the -5 to +5 segment there is less preference for acidic residues over basic residues. All 20 amino acids, except for cysteine, occur within the -5 to +5 segment. The closest cysteine residue to any of the

[1] Further details of this program and its general applicability for analyzing protein sequences will be described elsewhere (Folz, R.J. and Gordon, J.I., manuscript in preparation).

TABLE I

SEQUENCES ADJACENT TO TYROSINE SULFATE RESIDUES

1)	-LQIEVTVKGHVEYTMEANEDYEDYEYDELPAKDDPDAPLQPVTPLQ-
2)	-PRGDKLFGPDLKLVPPMEEDYPQFGSPK
3)	-FHKENTVTNDWIPEGEEDDDYLDLEKIFSEDDDYIDIVDSLSVSPTDSDVSAGN-
4)	-SRRWAIHTSEDALDASELEHYDPADLSPTESSLDLLGLNRT-
5)	-RKLVQAYQQRYNLQPYETTDYSNEEQSQRSSSEEQQTQRRK-
6)	-EGTPKPQSHNDGDFEEIPEEYLQ
7)	pQFPTDYDEGQDDRPKVGLGARGHRPY
8)	-HLVADPSKKQGPWLEEEEEAYGWMDFGRRSAEDEN
9)	-GAPQQREANDERRFADGQQDYTGWMDFGRRDDEDDVNERDV-
10)	-VSMIKNLQSLDPSHRISDRDYMGWMDFGRRSAEEYEYTS

Amino acid sequences extending 20 residues in both directions from sites of sulfation are presented using the single letter code. Sulfated tyrosine residues are underlined. The symbol pQ represents a pyroglutamyl residue. Sources of data on the amino acid sequences of sites of sulfation are shown below:
1) Fourth component of complement (C4). Human (4. 17. 18)
2) Alpha-2-Antiplasmin. Human (7. 8)
3) Heparin cofactor II. Human (9. 19)
4) Coagulation factor X. Bovine (20)
5) Yolk protein 2. Drosophila melanogaster (16. 21)
6) Hirudin, Leech (Hirudo medicinalis)(22)
7) Fibrinogen. Bβ-chain. Bovine (10. 23)
8) Progastrin. Human (24. 25)
9) Procaerulein IV. Frog (Xenopus laevis). (contains up to 4 copies of caerulein)(26)
10) Procholecystokinin. Porcine (27. 28)

sites of sulfation is located more than 20 residues away. Possible significance of the striking absence of cysteine residues is discussed below.

The hydropathy and secondary structure of sites of sulfation were analyzed by computational techniques described in Methods. As expected, considering the abundance of highly polar acidic amino acids adjacent to sites of sulfation, these sites have a very low average hydropathy value. This is especially pronounced in the segment immediately preceding the sulfated tyrosine residue. This segment appears as a prominent valley in a plot of hydropathy values (Fig. 1). The low hydropathy of sites of sulfation may be an important characteristic, because sulfation is a post-translational modification of proteins. Sites will be accessible to the action of a sulfotransferase only if they are exposed on the surface of a protein. The analysis of the distribution of amino acid residues suggests that sites of sulfation are not selected simply on the basis of low hydropathy, however. If that were the major factor involved in the selection of sites of sulfation, a high abundance of

TABLE 2
DISTRIBUTION OF AMINO ACIDS AROUND SITES OF SULFATION

Amino Acid	Position Relative to Site of Sulfation													Sum	Sum (-5 to +5)
	-12	-10	-8	-6	-4	-2	0	2	4	6	8	10	12		
Asp	0	5	3	1	1	1	1	1	4	2	9	3	3	57	30
Glu	1	1	2	2	1	2	2	5	7	3	7	4	3	54	36
TyrSO4	0	0	0	0	0	0	0	1	0	1	2	0	15	23	23
Lys	1	0	0	1	1	0	0	0	0	0	0	0	0	9	1
Arg	0	1	0	1	2	1	2	1	0	0	1	0	0	17	2
His	1	1	0	0	0	1	0	0	0	0	0	1	0	4	1
Ala	0	0	0	2	0	1	2	1	1	1	0	1	0	16	6
Asn	2	1	0	1	1	0	1	0	0	1	0	0	0	8	2
Cys	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gln	1	1	0	0	0	1	0	1	0	1	1	0	0	11	7
Gly	2	0	1	2	0	1	1	0	1	0	0	0	1	17	6
Ile	0	0	0	1	0	1	0	1	1	0	0	0	0	7	4
Leu	1	1	2	0	3	1	0	0	0	1	0	0	2	19	7
Met	1	1	1	1	0	0	1	0	1	0	0	0	0	10	5
Phe	0	0	1	0	1	2	1	0	1	0	0	0	0	11	3
Pro	1	0	0	2	1	0	2	1	0	2	0	0	0	21	6
Ser	2	0	0	0	1	0	1	2	2	0	0	0	0	25	8
Thr	1	0	1	0	0	1	0	0	0	1	2	0	0	11	6
Trp	0	1	1	0	1	0	0	0	0	0	0	0	0	6	3
Tyr	0	1	1	0	1	0	0	1	0	0	0	0	0	4	1
Val	0	1	0	0	1	0	0	0	0	0	0	0	0	5	1
Acidic Residues	1	6	5	3	2	3	3	7	8	8	11	13	6	134	89
Basic Residues	2	2	0	2	3	2	2	1	0	0	1	1	0	30	4

	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Asp	1	1	1	4	2	4	1	1	1	1	1	1	1	1
Glu	1	0	2	0	1	3	1	1	1	1	1	1	1	1
TyrSO4	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lys	1	1	1	1	0	1	0	1	1	1	1	1	1	1
Arg	0	0	0	0	0	0	0	1	1	1	1	1	1	1
His	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ala	2	2	0	0	0	0	0	0	0	0	0	0	0	0
Asn	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cys	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gln	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gly	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ile	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Leu	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Met	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Phe	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pro	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ser	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Thr	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Trp	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tyr	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Val	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Acidic Residues	2	1	3	4	2	7	1	2	2	2	2	2	2	2
Basic Residues	1	1	1	1	0	1	0	2	2	2	2	2	2	2

basic and other polar residues as well as acidic residues would occur near sulfation sites. Furthermore, studies using synthetic peptides as the substrates for protein tyrosine sulfotransferase indicate that acidic residues increase the affinity of peptides for the enzyme (15).

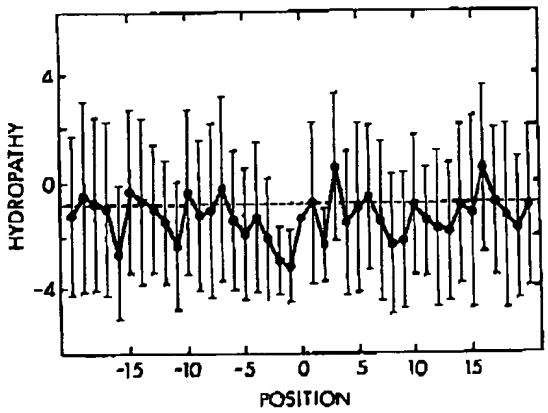

Fig. 1. Plot of average hydropathy values around sites of sulfation. Average hydropathy values at each position relative to sites of sulfation (Position 0) were calculated for the amino acid sequences shown in Table 1. Error bars indicate the standard deviation of all values at a given position. The reference line across the middle of the diagram corresponds to the mean hydropathy value of the 20 different amino acids which occur naturally in proteins.

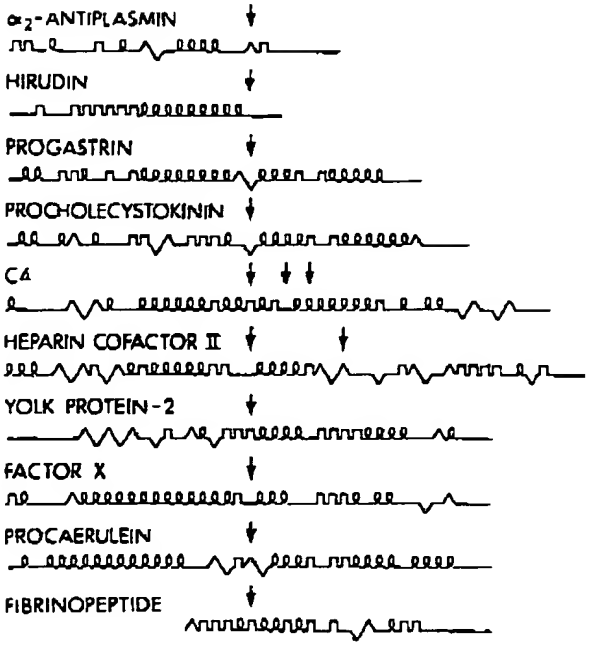

Fig. 2. Predicted secondary structure around sites of sulfation. Secondary structures of the amino acid sequences in Table I were determined using the method of Chou and Fasman (12). Arrows indicate sites of sulfation. Each symbol represents a single amino acid residue. Residues occurring in α-helices are shown as coils, β-sheets as zig-zags, β-turns as rectangular steps, and residues without predicted secondary structure are indicated as a horizontal line.

The secondary structure around each site of sulfation in the database was analyzed using the method of Chou and Fasman (12)(Fig. 2). This approach predicted that, many of the proteins will contain α-helical segment immediately before and after sulfation sites. However, tyrosine residues have a low propensity to form α-helices. As a consequence, some of the sites of sulfation are predicted to be located within short gaps between two helical segments. Only one sulfation site (the third site in C4) was predicted to lie within a helical segment, although 42% of the residues in the database were predicted to be in α-helices. That one example was the only case in which a site of sulfation was predicted to be within an extended segment (greater than three residues) of α-helix or β-sheet. Tyrosine residues favor the formation of β-sheet, but there was very low propensity to form extended β-sheets encompassing sites of sulfation. All but three of the sites of sulfation were closely preceded by α-helical segments. In two of the exceptions, yolk protein-2 and the first sulfation site in procholecystokinin, helical segments closely followed the sulfation site. The general conclusions suggested by predictions of secondary structure are that sulfation sites occur within short segments which lack extended secondary structure or are part of β-turns and that sulfation sites are closely flanked by α-helices. Similar conclusions

were reached by using the Para-Site program to average all amino acid sequences in the database and to generate single values at each position for the propensities to form α-helix, β-sheet, and β-turn conformations. Occurrence of sulfation sites at β-turns or at positions lacking secondary structure may contribute to accessibility of these sites. The significance of the probable occurrence of α-helices adjacent to most sulfation sites is not clear.

A number of factors may contribute to maximal exposure of sulfation sites at the surface of proteins. As already noted, known sulfation sites are bounded by multiple acidic amino acid residues, have low hydropathy, and lack extended secondary structure. In addition, a considerable proportion of sulfation sites, 7 out of the 15 in the database, occur within 20 residues of the termini of proteins. Furthermore, there is a complete absence of cysteine residues near sulfation sites. This may be important in permitting optimal access to segments that contain sites of sulfation. Formation of disulfide bonds with other segments of the polypeptide chain would introduce steric hindrance and restrict the flexibility of sites of sulfation. Extrapolation of the principle that accessibility is one of the most important characteristics of sites of sulfation suggests that sulfation sites may occur in extended segments of proteins in preference to globular domains.

Based on the foregoing analysis of amino acid sequences surrounding sulfation sites, five simple rules were empirically derived to aid in predicting the location of sites of sulfation. Tyrosine residues that are likely sites of sulfation are identified by the following criteria:

1) There is an acidic residue at position -1 or -2.
2) There are at least 3 acidic amino acid residues within 5 residues (positions -5 to +5) of the tyrosine residue.
3) No more than 1 basic amino acid residue are within 5 residues of the tyrosine.
4) No more than 3 hydrophobic residues (Ile, Leu, Phe, and Val) are within 5 residues of the tyrosine.
5) No cysteine residues are within 15 residues of the tyrosine.

Most sites of sulfation are expected to conform to all five of the criteria above. There are three known examples, canine fibrinopeptide B, bovine gastrin, and feline gastrin, in which sulfation sites conform with only four of the criteria (10). In those three cases, acidic residues are absent from the -1 and -2 positions. Application of these rules correctly identifies the 3 tyrosine sulfate residues in the fourth component of complement (4) and the 2 in heparin cofactor II (9) and excludes the other 59 tyrosine residues in these two proteins. Moreover, the rules correctly predict the absence of tyrosine sulfate in human serum albumin, which contains 18 tyrosine residues. Two sites of sulfation in yolk protein

2 are predicted by the above rules. but only one site was found experimentally (16). Some sites identified as potential sites of sulfation may not be sulfated due to steric hindrance of sites by higher order structure of the protein or by oligosaccharide chains. This would parallel the situation with N-linked glycosylation, in which not all potential sites identified by amino acid sequence are glycosylated. It is unlikely that any predictive method based solely on the amino acid sequence of proteins will perfectly predict sites of sulfation. Nevertheless, these rules should aid considerably in identifying sites of sulfation in proteins known to contain tyrosine sulfate and in predicting which proteins contain tyrosine sulfate. Identification of sites of sulfation may be essential for complete understanding of the structure and function of many proteins. The effect of sulfation on the biological activity of proteins has not been determined. but sulfation is known to have a profound effect on the activity of some peptides such as cholecystokinin (Refs. in 4).

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EXHIBIT 2

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TYROSINE SULFATION AND THE SECRETORY PATHWAY

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INTRODUCTION

In addition to proteolytic processing and carboxy-terminal amidation, which are reviewed elsewhere in this volume, neuropeptide processing involves various covalent modifications of amino acid side chains. Glycosylation, phosphorylation, and sulfation are the major such processing reactions. This review focuses on the sulfation of tyrosine, the only amino acid residue of neuropeptides shown to undergo this modification. Tyrosine sulfate is not specific for neuropeptides but has been found in many other proteins as well. Consequently, this review is not restricted to tyrosine sulfation of neuropeptides but includes general aspects of protein tyrosine sulfation that are relevant to this topic. The current status of the field is described in four parts, which deal with the occurrence of tyrosine sulfation, the characteristics of tyrosine-sulfated proteins, the subcellular localization and properties of the sulfating enzyme, and functional aspects of this modification. A comprehensive review and a short summary of the field have recently appeared (42, 43).

OCCURRENCE OF TYROSINE-SULFATED PROTEINS

Work during the past seven years has established that tyrosine sulfation is an ubiquitous posttranslational modification that occurs in essentially all animal cells containing a Golgi apparatus (35, 40, for review see 43). Up to 1% of the tyrosine residues of the total protein in an organism can be sulfated (9). Thus, sulfation is the most common posttranslational modification known for this amino acid.

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Species Distribution

Methods for detecting tyrosine sulfate in proteins have been described in detail (9, 19, 41). Tyrosine-sulfated proteins have been found in every investigated invertebrate and vertebrate species within the animal kingdom (for review see 43). Protein tyrosine sulfation has also been observed in the green alga *Volvox carteri* (W. B. Huttner, S. Wenzl, M. Sumper, unpublished; 76), which raises the possibility that this modification may be widespread in plants. No conclusive evidence has yet been obtained for the occurrence of tyrosine sulfate in proteins of unicellular eukaryotic organisms and prokaryotes. The data obtained so far suggest that during evolution, tyrosine sulfation of proteins first appeared consistently in multicellular eukaryotic organisms.

Tissue Distribution

Tyrosine-sulfated proteins have been found in all animal tissues studied and in all primary cell cultures and cell lines examined (35, 40, for review see 43). Different cell types contain characteristic sets of tyrosine-sulfated proteins, which suggests that proteins with cell type–specific expression are the main targets for tyrosine sulfation. From the work carried out so far it can be concluded that protein tyrosine sulfation occurs in both single cells and cells that are part of a tissue, in differentiated and undifferentiated cells, and in both normal and transformed cells.

CHARACTERISTICS OF TYROSINE-SULFATED PROTEINS

Intracellular and Extracellular Localization

The vast majority of tyrosine-sulfated proteins have not yet been characterized but are only known as sulfated bands on SDS polyacrylamide gels. The tyrosine-sulfated proteins characterized with regard to both localization and function are listed in Table 1. All of these proteins are synthesized by membrane-bound ribosomes in the rough endoplasmic reticulum, and most of them are secretory. Tyrosine sulfate has been found in constitutive as well as regulated secretory proteins.

The frequent occurrence of tyrosine sulfate in secretory proteins is consistent with results obtained with rats in vivo showing that plasma proteins contain much more tyrosine sulfate than tissue proteins (35). In addition, analysis of proteins transported in neurons by fast axonal transport showed that tyrosine sulfate is predominantly found in proteins delivered to nerve terminals, the site of secretion of such proteins (72), and that these tyrosine-sulfated proteins constitute the bulk of the total protein-bound tyrosine sulfate synthesized by neurons (S. B. Por & W. B. Huttner, unpublished data).

Table 1 Tyrosine-sulfated proteins

Protein	Species	Localization[a]	Reference
α-2-antiplasmin	man	sec	36
α-fetoprotein	man	sec	57
α-2-macroglobulin	rat	sec	35
aminopeptidase N	pig	plm	20
caerulein	frog	sec	4
cholecystokinin (CCK)	dog, man, pig	sec	61
C-terminal peptide of pro-CCK	man, rat, pig	sec	1, 21
complement C4 (α-chain)	man, mouse	sec	38, 49
dermatan sulfate (core protein)	man	sec	—[b]
entactin/nidogen	mouse	sec	66
factor X	cow	sec	59
fibrinogen	various species	sec	15, 35, 46
fibronectin	hamster, man, rat,	sec	56, 65
gastrin	various mammals	sec	5, 27
heparin cofactor II	man	sec	39
hirudin	leech	sec	67
immunoglobulin A (α-chain)	mouse	sec	—[c]
immunoglobulin G 2a (γ-chain)	mouse	sec	8
immunoglobulin M (μ-chain)	mouse, rat	sec	11
leucosulfakinin	cockroach	sec	62
leu-enkephalin	various mammals	sec	73
maltase-glucoamylase	pig	plm	20
phyllokinin	frog	sec	3
procollagen type III	man	sec	48
procollagen type V	chicken	sec	22
secretogranin I (chromogranin B)	cow, rat	sec	53, 70
secretogranin II	cow, rat	sec	53, 69, 70
S-protein/vitronectin	man	sec	—[d]
SG70	*Volvox*	sec	76
sucrase-isomaltase	pig	plm	20
thyroglobulin	mouse, pig, rat	sec	30, 35
yolk protein 1	fruit fly	sec	9
yolk protein 2	fruit fly	sec	9
yolk protein 3	fruit fly	sec	9

[a] Abbreviations: sec, secretory; plm, plasma membrane
[b] H. Kresse, personal communication
[c] P. A. Baeuerle, W. B. Huttner, unpublished
[d] A. Hille, D. Jenne, K. Stanley, W. B. Huttner, unpublished

Recent work indicates that tyrosine sulfation occurs in a wider variety of proteins derived from the rough endoplasmic reticulum than was initially assumed (44). A quantitative study (34) has shown that although most (65–95%) of the total protein-bound tyrosine sulfate synthesized by cells is recovered in secreted proteins, significant amounts of tyrosine sulfate are found in nonsecretory proteins. Several proteins tightly associated with mem-

branes have been found to contain tyrosine sulfate. Some of these [p61 of A431 cells (58); microvillar enzymes of intestinal epithelial cells (20)] are cell surface proteins, whereas others [p150 of the human hepatoma cell line HepG2 (34)] appear to be confined to intracellular membranes. Moreover, a soluble 45-kd glycoprotein of fibroblasts and HepG2 cells that is retained intracellulary in a membrane-enclosed compartment undergoes tyrosine sulfation (33). In contrast to oligosaccharide sulfation (18a), tyrosine sulfation has so far not been reported for lysosomal proteins, but this could be a problem of detection. Proteins not originating from the rough endoplasmic reticulum, such as cytoplasmic, nucleoplasmic, and mitochondrial proteins, do not become tyrosine sulfated under physiological conditions (43, 44).

Functions

The identified tyrosine-sulfated proteins are functionally diverse (see Table 1). In addition to neuropeptides, which constitute one major functional class, the list of identified tyrosine-sulfated proteins includes cell surface enzymes, proteins of the blood clotting system, of the extracellular matrix, of the immune system, and others. The biological significance of tyrosine sulfation is understood only in the case of a few neuropeptides, as will be discussed below.

TYROSYLPROTEIN SULFOTRANSFERASE

Tyrosylprotein sulfotransferase catalyzes the sulfate transfer from 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to tyrosine residues of proteins:

$$\text{protein} + \text{PAPS} \rightarrow \text{tyrosine-sulfated protein} + \text{PAP}$$

Such an enzymatic activity was first described in PC12 cells, a rat neuroendocrine cell line (53). This enzyme was found to have the correct substrate specificity since it catalyzed the tyrosine sulfation of secretogranin I and secretogranin II, the major tyrosine-sulfated proteins of PC12 cells (53, 70). Subsequent studies have demonstrated that tyrosylprotein sulfotransferase catalyzes the sulfation of a variety of endogenous and exogenous protein substrates in homogenates, membrane fractions, and extracts of numerous cells and tissues, including adrenal medulla tissue, fibroblasts, and AtT-20 cells (54, 55), BHK cells (26), brain tissue (74), A431 cells (58), neuroblastoma-glioma hybrid cells (13), and hybridoma cells (11). Moreover, tyrosylprotein sulfotransferase has been found in membrane fractions of all rat tissues examined (63).

Subcellular Localization

Tyrosylprotein sulfotransferase, as determined by subcellular fractionation, is localized to the Golgi complex (55). The active site of the enzyme is oriented

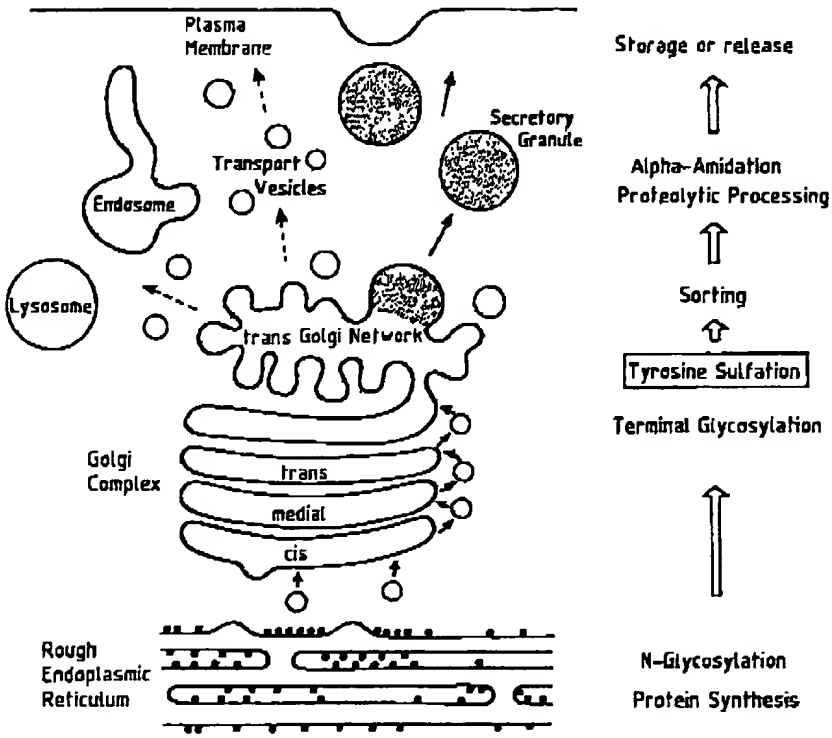

Figure 1 Tyrosine sulfation, neuropeptide processing, and the secretory pathway. The transport route of neuropeptides along the secretory pathway is indicated by solid arrows. Dashed arrows indicate transport routes not normally taken by neuropeptides. The processing steps are depicted on the right next to the compartments in which they occur, and their sequence is indicated by open arrows.

towards the lumen of the Golgi (55), as one would expect in view of the topology of tyrosine-sulfated proteins. A specific transmembrane carrier system for PAPS from its site of synthesis, the cytosol, to the Golgi lumen supplies the enzyme with its nucleotide cosubstrate (71). Within the Golgi complex, tyrosylprotein sulfotransferase acts specifically in the *trans* Golgi (see Figure 1). This conclusion comes from a study on immunoglobulin M (11) in which the addition of sulfate to tyrosine residues during intracellular transport was compared with the well-characterized steps of processing of N-linked oligosaccharides. Tyrosine sulfation was found to take place in the same compartment as galactosylation and sialylation (i. e. the *trans* Golgi), occurring (at least in part) shortly after these terminal glycosylation reactions. Thus tyrosine sulfation is the last known covalent addition to proteins before their exit from the *trans* Golgi. Tyrosine sulfation has not been found to occur in any other compartment that is part of, or derived from, the secretory pathway. Precursors of tyrosine-sulfated secretory proteins isolated from the rough endoplasmic reticulum and from the *cis* and medial Golgi are not yet tyrosine sulfated (11, 23, 52). Subcellular fractions enriched in lysosomes or

secretory granules do not contain significant amounts of tyrosylprotein sulfotransferase activity (55).

The localization of tyrosylprotein sulfotransferase in the *trans* Golgi, together with the notion that proteins secreted by either constitutive or regulated pathways contain tyrosine sulfate, suggests that tyrosine sulfation precedes the sorting of secretory proteins to these pathways, a process thought to occur at the exit site of the Golgi complex (see 29, 50). In the cases where neuropeptides are sorted as precursors and processed proteolytically in secretory granules, tyrosine sulfation precedes, and thus may affect, proteolytic processing of neuropeptide precursors (see Figure 1). In these cases the precursors of neuropeptides rather than the cleaved neuropeptides are the physiological substrates for tyrosylprotein sulfotransferase.

Properties

Tyrosylprotein sulfotransferase has all the characteristics of an integral membrane protein. It is not extracted from membranes by low ionic strength, high ionic strength, or pH11 treatment but is solubilized by nonionic detergents and partitions into the detergent phase upon Triton X-114 extraction and phase condensation (55, 63). Tyrosylprotein sulfotransferase solubilized from Golgi membranes of bovine adrenal medulla has been studied with an amino acid polymer, $(Glu^{62}, Ala^{30}, Tyr^{8})_n$ (55), with tubulin (55), and with various synthetic peptides (63) that correspond to tyrosine sulfation sites in the cholecystokinin precursor (1, 21) and in secretogranin I (14).

The solubilized enzyme from adrenal medulla has a pH optimum between 6.0 and 6.5 (55). This property fits well with the *trans*-Golgi localization of the enzyme since the *trans* cisternae of the Golgi are known to have a slightly acidic pH (7). With both endogenous and exogenously added substrate proteins, tyrosylprotein sulfotransferase activity is stimulated by the divalent cations Mg^{2+} and Mn^{2+} and is inhibited by EDTA (53, 55). A pH optimum of ~6 and stimulation by Mn^{2+} have also been observed for the nonsolubilized enzyme from rat brain microsomes (74), which has recently been found (75) to be very similar to the enzyme originally characterized in adrenal medulla (54, 55). In contrast, sulfation of an endogenous membrane protein in A431 cells was not inhibited by EDTA (58). The apparent K_m of tyrosylprotein sulfotransferase for various synthetic peptide substrates is in the micromolar range; the lowest value reported is 0.3 μM for $(Glu^{62}, Ala^{30}, Tyr^{8})_n$ (55, 63, 74, 75). The apparent K_m of solubilized tyrosylprotein sulfotransferase for the cosubstrate PAPS is 5 μM (55).

Consensus Features of Tyrosine Sulfation Sites

The sequences surrounding sulfated tyrosine residues are known for several neuropeptides and other secretory proteins. By comparing these sequences,

consensus features of tyrosine sulfation sites have been deduced and criteria for predicting tyrosine sulfation sites have been proposed (37, 43, 44; see Table 2).

PRESENCE OF ACIDIC AMINO ACIDS Sulfated tyrosine residues are usually surrounded by several acidic amino acid residues. Most frequently, an aspartic or glutamic acid is found at position −1 of the sulfated tyrosine and at least two more acidic residues are present between positions −5 and +5 (see footnote to Table 2). The hypothesis (53) that acidic amino acid residues in the vicinity of tyrosine residues are involved in the recognition of substrate proteins by tyrosylprotein sulfotransferase has received experimental support by the demonstration that polymers of tyrosine and glutamic acid are sufficient as substrates for sulfation (55) and by the observations that all of the synthetic peptides found to serve as in vitro substrates (13, 63, 74) contain acidic amino residues, including one in position −1. In contrast to the abundance of acidic amino acids, basic amino acids are rarely found in the vicinity of tyrosine sulfate residues (not more than one basic residue between positions −5 and +5).

PRESENCE OF TURN-INDUCING AMINO ACIDS All known tyrosine sulfation sites contain amino acids with the potential to induce turns in the polypeptide. At least one proline or glycine, the amino acids with the strongest turn-inducing potential (64), or at least two of the three other amino acids with significant turn-inducing potential [aspartic acid, serine, and asparagine (64)]

Table 2 Consensus features of tyrosine sulfation sites[a]

Acidic amino acids	Presence of an acidic amino acid at position −1 and of at least three acidic amino acid residues from −5 to +5. Paucity of basic amino acid residues (not more than one from −5 to +5).
Secondary structure	Presence of turn-inducing amino acid residues from −7 to −2 and from +1 to +7 (at least one pro or gly or at least two asp, ser or asn).
Exposure on protein surface	Paucity of hydrophobic amino acid residues (not more than three from −5 to +5).
Steric hinderance	Absence of disulfide-bonded cysteine residues from −7 to +7. Absence of N-linked glycans near the tyrosine.

[a] For details see References 37, and 43. Negative numbers refer to the sequence position of residues at the N-terminal side of the sulfated tyrosine and positive numbers to those at the C-terminal side. The sulfated tyrosine itself is position 0.

are found in all sulfation sites within positions −7 to −2 and +1 to +7 of the tyrosine sulfate residues. The presence of turn-inducing amino acids probably exposes tyrosine residues located near acidic amino acids, thereby facilitating their access to the active site of tyrosylprotein sulfotransferase. Likewise, the scarcity of hydrophobic amino acids in the vicinity of tyrosine sulfate residues presumably reflects the need to expose tyrosine sulfation sites on the surface of substrate proteins.

ABSENCE OF DISULFIDE BONDS AND N-LINKED GLYCANS No known tyrosine sulfation site contains any cysteine residue between positions −7 and +7. Most cysteine residues in secretory proteins are involved in formation of disulfide bonds (which are formed in the rough endoplasmic reticulum and thus before the protein reaches the compartment of sulfation). This raises the possibility that disulfide bonds in the vicinity of a tyrosine residue prevent sulfation even if the latter is located in a sequence containing acidic amino acids and turn-inducing amino acids.

No known tyrosine sulfation site is located near an N-glycosylation site (Asn-X-Ser or Asn-X-Thr; see 51). It is likely that N-linked oligosaccharides (which are added to the protein prior to sulfation) prevent sulfation of a nearby tyrosine because of steric hinderance, even if the latter is located in a sequence containing acidic amino acids and turn-inducing amino acids. In mouse IgG2a, a tyrosine residue preceded by two acidic amino acids (tyr 179) adjoins the N-glycosylation site of the constant part of the heavy chain. After inhibition of N-glycosylation, the IgG2a heavy chain becomes tyrosine-sulfated in the constant region (8), presumably at tyr 179, which is now accessible to the tyrosylprotein sulfotransferase.

Lack of Reversibility of Tyrosine Sulfation in Vivo

The available evidence indicates that tyrosine sulfation is poorly reversible or even irreversible in vivo. For various secretory proteins (e.g. IgM and proteins secreted by fibroblasts) it has been shown that essentially all of the tyrosine sulfate present in the intracellular forms of these proteins at the end of a labelling pulse is recovered when all the labelled secretory proteins had been chased into the medium (11, 34). Certain secretory proteins isolated from secretory granules, e.g. secretogranin I (14), are stoichiometrically tyrosine sulfated. Hence, no significant protein desulfation occurs during transport to the cell surface or in secretory granules. This indicates either the absence of a sulfotyrosylprotein sulfatase or the lack of contact between such an enzyme, if one exists, and the tyrosine-sulfated proteins studied.

The fate of protein-bound tyrosine sulfate after secretion has been investigated in vivo in the case of fibrinogen. The half-life of sulfate-labelled fibrinogen in vivo is the same as that of amino acid–labelled fibrinogen, which indicates that no desulfation of this protein occurs after secretion in the

living animal (16). After injection of sulfate-labelled fibrinopeptide B into rabbits, essentially all of the sulfate label was recovered in the urine in the form of free tyrosine sulfate and its deaminated metabolites. This result shows that even after degradation of this tyrosine-sulfated protein there is no significant desulfation (47). This may be true for other tyrosine-sulfated proteins as well, since the amounts of tyrosine sulfate excreted in the urine are too high to be accounted for only by the turnover of fibrinogen (31).

FUNCTIONAL ASPECTS OF TYROSINE SULFATION

The biological role of protein tyrosine sulfation has been established only in the cases of a few neuropeptides. This is partially due to the fact that studies comparing sulfated and unsulfated forms of proteins (rather than small peptides) are experimentally more difficult; the unsulfated form of a tyrosine-sulfated protein could not easily be obtained. Recently, however, the sulfate analogue chlorate has been shown to be a potent inhibitor of protein sulfation in intact cells (10). The availability of an inhibitor of sulfation will greatly facilitate functional studies on protein tyrosine sulfation.

Biological Activity of Neuropeptides

Tyrosine sulfation is essential for the biological activity of certain neuropeptides. For example, the hormonal activity of cholecystokinin (CCK) has been shown to depend on the sulfation of the tyrosine residue (60). Sulfated CCK has been found to be 260 times more potent than unsulfated CCK (2, 17). In contrast to the positive effect of sulfation on the hormonal activity of CCK, the biological activity of leu-enkephalin is inhibited by tyrosine sulfation (73).

Diversification of Translation Products

Tyrosine sulfation can be a means of producing more than one phenotype from a single translation product. This possibility for functional diversification is indeed utilized by gastrin-producing cells, as shown by Brand et al (18). Gastrin requires tyrosine sulfation for its pancreatic secretagogue activity (45), whereas its ability to stimulate gastric acid secretion is unaffected by sulfation (28). Thus a second biological activity of a single translation product results from tyrosine sulfation. The substoichiometric tyrosine sulfation observed in certain proteins, e.g. IgM (11) and factor X (59), may serve a similar purpose in creating subpopulations of molecules that are functionally altered.

Proteolytic Processing

Tyrosine sulfation can affect the sensitivity of specific sites in proteins to proteolytic cleavage. For example, chymotryptic cleavage in vitro does not

occur at the C-terminal side of sulfated tyrosine residues in caerulein and in yolk protein 2 of the fruit fly (12). With regard to a proteolysis-promoting effect, an intriguing correlation has been observed with gastrin (6, 68). The processing of the gastrin precursor varies in different tissues. The extent of processing toward the smallest product correlates with the extent of tyrosine sulfation. Since tyrosine sulfation probably precedes the proteolytic processing of peptide precursors (see Figure 1), one may speculate that sulfation promotes the processing of the gastrin precursor.

Intracellular Transport of Secretory Proteins

A possible role of tyrosine sulfation in the intracellular transport of a secretory protein has been investigated by site-directed mutagenesis. Vitellogenin 2 of *Drosophila melanogaster* is stoichiometrically sulfated at tyrosine 172 (9, 12). After mutagenesis of tyrosine 172 to phenylalanine, the wild-type and mutated vitellogenin 2 were expressed in fibroblasts, and their sulfation and secretion were studied (24, 25). The wild-type protein was sulfated at the same residue as the vitellogenin 2 synthesized in the fly, whereas the mutated protein produced by fibroblasts was not sulfated (24). The unsulfated vitellogenin 2 was still secreted (24); however, the kinetics of secretion of the unsulfated vitellogenin 2 were markedly reduced compared to those of the sulfated form (25) and suggested an at least twofold slower passage of the unsulfated protein through the *trans* Golgi.

SUMMARY

Tyrosine sulfation is a widespread posttranslational modification. Most tyrosine-sulfated proteins identified so far are secretory, including several neuropeptides. Tyrosine sulfation occurs in the *trans* Golgi and is one of the last processing steps before proteins exit from the Golgi complex. The sulfation reaction is catalyzed by tyrosylprotein sulfotransferase, an integral membrane protein that recognizes tyrosine residues in exposed protein domains containing acidic amino acids. In the cases studied to date, tyrosine sulfation has been found to be irreversible, resulting in a life-long alteration in the phenotype of the secretory proteins. The biological role of tyrosine sulfation has so far been elucidated in only a few cases. The intracellular transport kinetics of a secretory protein and the biological activity of certain neuropeptides have been found to be affected by this modification. Future functional studies will be greatly facilitated by the use of chlorate, a sulfate analogue that has recently been found to be a potent and nontoxic inhibitor of sulfation in intact cells.

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